MOLECULAR PHYLOGENY OF *TRIBOLIUM* (DANTHONIOIDEAE: POACEAE) AND ITS TAXONOMIC IMPLICATIONS

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ABSTRACT

Molecular sequence data from two noncoding regions of DNA (ITS AND TRNL-F) Were used to produce a phylogeny for the genus *Tribolium* and its African and Australasian allies. Topological comparisons of the combined molecular data with the appended existing morphological tree were made. A significant incongruence was revealed. Molecular data indicate that *Tribolium* is paraphyletic. The formally defined sections: Uniolae, Acutiflorae and Tribolium have been retrieved by the molecular data. The analysis retrieved *Karroochloa* as polyphyletic. Monophyly of *Schismus* is strongly supported. The Australasian species form a monophyletic clade. Data support early divergence of the *Merxmuellera* species and *Pseudopentameris macrantha.*

INTRODUCTION

Tribolium is a genus of annual and perennial temperate, C_3 grasses, currently comprising a total of ten species (Linder and Davidse, 1997). The genus is restricted to southern Africa, where the species are endemic to the winter rainfall region of the western Cape, which includes the Cape Floristic and Namaqualand regions. Here it forms part of the fynbos and succulent Karoo floras, with marginal populations of the most widespread species occurring in the neighbouring biomes. Ubiquitous members occur within the full range of habitats available in these biomes and specialists have speciated into unique habitats (Linder and Davidse, 1997). Altitude and soil type differentiation seem to have been important in driving differentiation in the genus (Linder and Davidse, 1997).

Tribolium is a member of the danthonioid grasses. These grasses are characterised by the possession of haustorial synergids cells in the embyo (Verboom et al, 1994) and a basic chromosome number of x=6 (Spies and Visser 1994). Studies of the generic composition and relationship in danthonioid grasses (Barker et al. 2000; Barker et al. 2003) have identified seven core monophyletic groups in the danthonioid grasses on the basis of ITS, rpoC2 and rbcL sequence variation. The relationships among these groups remain uncertain due to differences in the way they are resolved by alternative markers (Barker et al, 2000). *Tribolium* is found in the so-called "*Rytidosperma*" clade. In addition to *Tribolium* this clade has been shown to comprise the African genus *Karroochloa* and some species of *Merxmuellera*, the African-Eurasian *Schismus*, and the Australasian genera *Rytidosperma*, *Austrodanthonia, Notodanthonia* and *Joycea* (Barker, Morton and Linder, 2000). A key synapomorphy of the *Rytidosperma* clade is a distinctive obovate caryopsis (Linder and Verboom, 1996). However, the monophyly of this clade is weakly supported by both ITS and rpoC2 (< 50% bootstrap) (Barker et al. 2000). Morphological data splits this clade into three smaller groups, Rytidosperma "A", "B" and "C", with Rytidosperma "A" which comprises Tribolium and Rytidosperma pumillum (Barker et al. 2000). In

addition, phylogenetic analysis of the morphological data suggests that *Tribolium* is closely related to *Schismus* (Linder and Verboom 1996, Linder and Davidse, 1997).

Previous phylogenetic studies based on morphology (Linder and Davidse 1997) and cytogenetic data (Visser and Spies 1994) support the monophyly of *Tribolium,* identifying six synapomorphies: these include contracted inflorescences, glabrous calli with horizontal joints, and acute, unlobed lemmas with the awns fused completely to the potential lemma lobes (Linder and Davidse, 1997). The effectiveness of these studies in tesing the monophyly of the genus is weak due to the small number of outgroups included. The analyses of Barker et al. (2000, 2003) based on ITS and rpoC_2 data each sampled two species of *Tribolium* (*T. pusillum*, + *T. uniolae* and T. pusillum + *T. hispidum*). Whereas ITS data retrieved a paraphyletic *Tribolium* with low support (< 50% bootstrap), the rpoC_2 data resolved the genus as monophyletic with 100% bootstrap support. These molecular tests of the monophyly of the genus are weak, however being based on a very small sample of the species.

The early taxonomic history of *Tribolium* was messy due to poor understanding of generic limits and widespread misapplication of names (Linder and Davidse, 1997). The situation stabilized after 1900, with most workers at this time assigning current-day *Tribolium* species to three genera: *Lasiochloa* Kunth, *Plagiochloa* Adamson and Sprague (=*Brizopyrum* sensu Stapf) and *Urochlaena* Nees (Table 1.). Renvoize (1985) united all the *Plagiochloa* and *Lasiochloa* species under an earlier name, *Tribolium*, recognising 11 species in total (Renvoize, 1984). Visser and Spies's (1994a, b, c, d, e) studies based on chromosome numbers and cytogenesis of meiotic chromosomes behaviour have contributed to the taxonomic studies and present delimitations of biological species within the presently recognised *Tribolium* genus. Subsequent cytogenetic studies Visser and Spies's (1994a, b, c, d, e) revealed that three of the *Tribolium* species (*T. uniolae*, *T. alternans* and *T. amplexum)* actually formed a hybrid swarm and these were thus reduced to a single species, *T. uniolae,* and the total number of species in *Tribolium* reduced to nine (Visser and Spies, 1994e). In their morphologically based study, Linder and Davidse (1997) found *U. pusilla* to be nested within a monophyletic *Tribolium*, and so transferred the species to the latter genus, raising the total number of species to ten. *Urochlaena pusilla* has all

the synapomorphies of *Tribolium,* and based on leaf anatomy and cytology, appeared to be closely related to *T. utriculosum* and *T. echinatum* (Linder and Davidse, 1997).

Table 1. Major taxonomic treatment of the genus *Tribolium*. Generic abbreviations: *B=Brizopyrum, L=Lasiochloa, T=Tribolium, U=Urochlaena P=Plagiochloa_*

Stapf (1900)	Adam and Spraque (1941)	Renvoize (1985)	Linder and Davidse (1997)
B. capensis	*P. uniolae*	*T. uniolae*	*T. uniolae*
B. alternana		*T. alternans*	
		T. amplexum	
B. brachystachya	*P. brachystachya*	*T. brachystachyum*	*T. brachystachyum*
B.obliterum	*P. oblitera*	*T. obliterum*	*T. obliterum*
L. obtusifolia		*T. obtusifolium*	*T. obtusifolium*
B. glomeratum			
B. acutiflorum	*P. acutiflora*	*T. acutiflorum*	*T. acutifolium*
B. ciliare	*P. ciliaris*	*T. ciliare*	*T. ciliare*
L. longifolia		*T. hispidum*	*T. hispidum*
L. utriculosa		*T. utriculosum*	*T. utriculosum*
L. ciliaris		*T. echinatum*	*T. echinatum*
U. pusilla		*U. pusilla*	*T. pusillum*

Infrageneric studies in *Tribolium* based on cytogenetic (Visser and Spies, 1994) and morphological (Linder and Davidse, 1997) data both retrieved three groups within the genus: section *Tribolium (T. ciliare, T. hispidum, T. utriculosum and T. pusillum*), section *Acutiflorae (T. acutiflorum, T. obliterum and T. obtusiflorum)* and section *Uniolae (T. brachystachyum and T. uniolae).* However, these are weakly supported, in large part because several of the characters unique to the genus do not have congruent distributions within it (Linder and Davidse, 1997). While Linder and Davidse (1997) found these internal groups to be monophyletic, Visser and Spies (1994) indicated section *Tribolium* to be paraphyletic. However, these groups are comparatively distinct morphologically, leading Linder and Davidse (1997) to formally describe them at sectional level.

The high degree of morphological character incongruence may be a product of convergence or hybridisation (Givnish and Sytsma 1997a). Molecules may be less prone to convergence and so give a clearer phylogenetic signal (Givnish and Sytsma 1997b). Molecular studies often yield gene trees that disagree in intergeneric relationships they support (Mason-Gamer and Kellogg, 1996) due to genome rearrangements or transference resulting from deletions, insertions, hybridisation or genetic exchange between nuclear and organellar genomes. When multiple data sources support the same topology, or the conflict between them is weak, Lee (1997) suggested that such data may be combined in a single analysis, but if there is evidence of significant differences in the phylogenetic hypotheses, data should not be combined, but be partitioned into separate analysis. In addition, molecular data generally provide more informative characters than nonmolecular data and a topology generated from their combined analysis will be biased by the larger data set (Hedges and Maxon, 1996). Maxson and Hedges (1996) suggest that molecular data be used for estimating the phylogeny and morphological data for mapping the evolutionary changes onto the resulting phylogeny. This is useful for identifying the synapomorphies that link the resulting monophyletic groups as Backland and Bremer (1998) have shown that in addition to being monophyletic, groups of taxa should be morphologically comparable in order to facilitate taxon diagnosis.

In view of the uncertainties surrounding the monophyly of *Tribolium* as well as the poor support for internal relationships within the sections, this study uses molecular

data, from both the plastid and nuclear genomes to test: 1) the monophyly of *Tribolium* and its relationship with its allies, especially the closely related genera such as *Schismus* and *Karroochloa*, and 2) the current hypothesis of interspecific relationships within *Tribolium*. Using these results allow critical assessment of the sectional taxonomy and generic limits proposed by Visser and Spies (1994) and Linder and Davidse 1997)

Backland and Bremer (1998) suggest that formally recognised groups of species should be monophyletic. Monophyletic groups are considered to be a natural product of evolutionary descent comprising all the descendents of a common ancestor. Paraphyletic groups should not be recognised formally in phylogenetic studies because they are likely to confound the study of evolution. Maxon and Hedges (1996) however pointed that in many cases paraphyly is inevitable, and morphologically well-defined groups exist and they should be formally recognised. Maxon and Hedges (1996) have also added that monophyletic groups should also be morphologically recognizable.

MATERIALS AND METHODS

Sampling

Taxon sampling was guided by higher level analysis of Barker et al. (2003) and included a full representation of *Tribolium* species as well as representatives of all closely related genera/clades. In particular I included all ten species of *Tribolium* (sensu Linder and Davidse, 1997), three *Schismus* species, three *Karroochloa* species, four *Merxmuellera* species, *Joycea pallida*, of *Notodanthonia gracilis, Austrodanthonia auriculata and Pseudopentameris macrantha.* Two *Rytidosperma* species (those included in the Rytidosperma clade by Barker et al. (2003) *Karroochloa curva curvá* and *Schismus pleuropogon,* and the trnL-F region of *R. pumillum* were omitted because they proved difficult to amplify. *Pentaschistis curvifolia* was included as an outgroup since both molecular evidence and the lack of a distinctive obovate caryosis places this species outside the *Rytidosperma* clade. A total of 27 species was sampled. Except for *T. brachystachyum* and *S. barbatus*, all sampled species were represented by a single accession. Most material was collected in the field with vouchers being deposited at BOL. Material of *Merxmuellera*, *P. Macrantha* and the Australasian taxa, was provided by N. P. Barker (Rhodes University, Grahamstown). ITS sequences for species *M. stricta*, *M. dura, M. disticha, P. macrantha* and *R. pumilum* were obtained from Genbank

Table 1. List of samples used for DNA sequencing

Genus	**Species name**	**Voucher no. ITS**	**Voucher no. trnL-F**
Karrochloa	*K. schismoides* Conert and Tuerpe	Verboom 562	Verboom 562
	K. tenella Conert and Tuerpe	Verboom 587	Verboom 587
	K. purpurea Conert and Tuerpe	Verboom 705	Verboom 705
Schismus	*S. barbatus* Conert and Tuerpe	Verboom 503	Verboom 503
	S. barbatus Conert and Tuerpe	Verboom 572	Verboom 572
	S. scaberrimus Conert and Tuerpe	Verboom 573	Verboom 573
	S. inermis Conert and Tuerpe	Verboom 586	Verboom 586
Tribolium	*T. acutiflorum* Linder and Davidse	Verboom 504	Verboom 504
	T. uniolae Linder and Davidse	Verboom 530	Verboom 530
	T. hispidum Linder and Davidse	Verboom 532	Verboom 532
	T. utriculosum Linder and Davidse	Verboom 568	Verboom 568
	T. echinatum Linder and Davidse	Verboom 576	Verboom 576
	T. pusillum Linder and Davidse	Verboom 588	Verboom 588
	T. brachystachyum Linder and Davidse	Verboom 593	Verboom 593
	T. ciliare Linder and Davidse	Verboom 596	Verboom 596
	T. obtusifolium Linder and Davidse	Verboom 597	Verboom 597
	T. obliterum Linder and Davidse	*Verboom* 598	Verboom 598
Merxmuellera	*M. stricta* Conert	Genbank AF019871	Linder 5497
	M. dura Conert	Genbank AF019872	Linder 5421
	M. disticha Conert	Genbank AF367600	NPB 1676
	M. guillamodae Conert	Barker	Barker
Notodanthonia	*N. gracilis* Zotov	Linder 5683	Linder 5683
Austrodanthonia	*A. auriculata* Linder	Linder 5569	Linder 5569
Joycea	*J. pallida* Linder	Linder 5564	Linder 5564
Pseudopentameris	*Ps. macrantha* Conert	Genbank AF367598	Linder 5470
Pentaschistis	*P. curvifolia* Philipson and Connor	Verboom 594	Verboom 594
Rytidosperma	*R. pumillum* Clayton and Renvoize	Barker AF019878	Linder 5747
	R. nudiflora Clayton and Renvoize	Linder 5693	Linder 5693

DATA

MOLECULAR DATA

Total DNA was extracted from silicagel-dried leaf material of each sample following the CTAB DNA extraction protocol, modified from Gawel and Jarret (1991) and using liquid nitrogen. The genomic DNA extracts were used as template for PCR amplification.

Initial PCR amplifications were done in 50ul reactions using an applied Biosystems genemp 2700 Thermocycler (Applied Biosystem, Foster city, CA, USA). Each reaction contained 32.7ul distilled water, 5ul 10X NH4 buffer, 5ul 50mM MgCl2 buffer, 2ul dNTPs, 1.5ul each of the forward and reverse 10mM primers, 0.3ul Taq polymerase and 2ul of diluted (1/10 or 1/100) DNA template. The PCR thermal profile used involved 30 cycles of: 1 minute denaturation of the double stranded template DNA at 94°C, 1 minute annealing of primers to single stranded template DNA at 52°C, and a 2 minute primer extension period at 72°C. The amplification regime also included the initial denaturation step of 3 minutes at 94°C and final extension of 7 minutes at 72°C.The entire trnL-F region of the plastid genome including the intergenic spacer between trnL and trnF as well as the trnL intron was amplified using the primers 'c' and 'f' described by Taberlet et al (1991). The complete ITS region of the nuclear ribosomal genome including the internal transcribed spacers, ITS1 and ITS2, and the 5.8S gene was amplified using primers ITS5 and ITS4 described by White et al (1990). Amplification products were verified by electrophoresing part of the sample on an ethidium bromide-containing agarose gel alongside a fragment size marker.
The remainder of the PCR products were purified using the QIAquick® Qiaqen™PCR purification kit (Qiagen GmbH, Hilden Germany) to separate PCR reagents from the desired PCR product. Purified products were also verified by the above electrophoresis procedure.

Cycle sequencing was done in10ul reaction mixtures. Each reaction contained 1.34ul distilled water, 3ul 2.5X buffer and 1.5ul Terminator Ready Reaction mix (both from

the ABI prism Big Dye kit) and 0.16ul 10mM primer. Between 1ul and 3ul volumes of the purified PCR products were used, with the balance made up with distilled water. The cycle sequencing used primers 'c', 'd', 'e', 'f' for trnL-F and ITS4 and ITS5 for ITS. The cycle sequencing thermal profile used was 25 cycles of: 30 seconds at 96 C, 15 seconds at 50 C and 4 minutes at 60 C. The cycle sequencing products were realised on an ABI PRISM® 3100 Genetic analyser (Applied Biosystems, Foster City, CA, USA) by the University of Stellenbosch, Matieland, South Africa.

Forward and reverse sequences were assembled and edited in Seqman (LaserGene system software, DNA Inc.), and the consensus sequences were saved as a single file. Sequences obtained from Genbank were added to the file. The consensus sequences were aligned manually in MegAlign (LaserGene System Software, DNAstar Inc.). The analysis required the insertion of gap characters so that homologous nucleotide base positions could be compared (Mindell, 1991). Variable and unambiguous sites were checked against the original traces. Where gaps occurred in the data they were treated as missing data in the nucleotide matrix. Potentially phylogenetically informative indels appearing in unambiguously aligned areas were coded in a separate binary matrix as presence/absence (Simmons and Ochoterena 2000: simple gap coding), being judged homologous where they were identical in length and position. Characters on the untrimmed ends of the sequences were not included in the analyses. The data partitions were put in a single matrix and a total of 1940 characters were obtained: ITS (738 characters + 8 gaps), trnL-F (1141 characters + 14 gaps), and morphology (39 characters).

Parsimony analysis using a heuristic search algorithm was performed in PAUP*4.0b10 (Swofford 1998) with all characters treated as unordered and of equal weight. Heuristic searches for most parsimonious tree/s were done using a random addition sequence with 10000 replicates using the tree-bisection-reconnection (TBR) branch-swapping algorithm, and with the 'Multrees' option in effect. For tree representation, cataschistic *P. curvifolia* was specified as the outgroup and the ingroup was specified as monophyletic. Data partitions were initially analysed separately, then ITS and trnL-F were analysed in a combined molecular analysis. When multiple most parsimonious trees were obtained, trees were summarised to a single strict consensus tree. Evaluation of character support for the nodes was done

using 500 bootstrap replicates based on a simple addition sequence with 'Maxtrees' set to 1000.

Topological conflict among the trees the ITS, and trnL-F and morphological and molecular trees was tested for statistical support using Wilcoxon Sign Rank (WSR) tests as applied by Templeton (1983) and Mason-Gamer and Kellogg (1996). When two data sets produce significantly conflicting topologies, Templeton tests are used to determine whether analysis of one data set under the constraints of the conflicting topology generated by the other data set results in a significantly less parsimonious tree than in the absence of constraints. The constraints may be based on the specific nodes of conflict individually or on the entire conflicting topology. Because the points of conflict can be tested individually, the method is able to locate significant points of conflict.

MORPHOLOGICAL DATA

Linder and Davidse's (1997) study used 39 characters which were coded for all species included in this study except four of *Merxmuellera* species and *Pseudopentameris macrantha*. To facilitate comparison between phylogenetic estimates based on molecules and morphology, morphological characters for these five species were coded from herbarium specimen housed at BOL. The resulting character matrix (provided in Appendix 1) was manually entered into a single matrix with other data partitions, the character number being identical to that used by Linder and Davidse (1997). Specimens of the Australasian species were not available for morphological character coding. Characters were treated as unweighted + unordered (Fitch 1970) except for seven characters which Linder and Davidse (1997) specified as additive. Parsimony searches were done for morphological data as outlined for molecular data. Characters were optimised onto the combined molecular tree using the describe Trees command in Paup. Both DELTRAN and ACCTRAN methods were used.

RESULTS

DATA SET INFORMATIVENESS AND CONSISTENCY

Lengths of the trnL-F sequences ranges from 728-1008 base pairs while the ITS sequences were 580-718 base pairs long. Alignment was largely straightforward but proved challenging in some areas especially in relation to *M. guillamodae* and *T. obliterum*. The topologies obtained from analysis of the separate and combined molecular data sets received moderately high CI indicating lower levels of homoplasy than that associated with the morphological data. The molecular data also show high resolving power, yielding nine well-supported nodes in the ITS, 13 in combined molecular trees and 12 well supported nodes in trnL-F (bootstrap≥80%). The morphological tree is poorly resolved.

Table 2: Statistical information describing most parsimonious trees produced by analyses of all data sets.
C.I=consistency index, R.I=Retention index, MPT=most parsimonious tree, PIC=parsimony informative characters

Data set	No. of MPTs	Tree Length	CI	RI	No. of PICs
trnL-F	491	1053	0.618	0.627	75
ITS	6	1023	0.649	0.673	45
Morphology	140	128	0.398	0.750	36
Combined molecular	12	987	0.601	0.599	200

MOLECULAR PHYLOGENY

ITS

Analysis of the ITS sequence data retrieved six most-parsimonious trees whose consensus is presented in Appendix Fig. 2. The tree resolves *Tribolium* as paraphyletic with respect to *K. purpurea*, *K. tenella* and a clade containing the Australasian species. Due to the early divergence of section Uniolae *K. purpurea* + *K. tenella* were resolved as sister to section Acutiflorae (bootstrap = 80%)and a monophyletic *Schismus* is resolved as sister to *K. schismoides* (bootstrap =100%), while *Karroochloa* is polyphyletic. Sections Uniolae and Acutiflorae are retrieved as monophyletic with strong bootstrap support (100% and 87% respectively) while section *Tribolium* is paraphyletic but with no bootstrap support. Basal clade of *Merxmuellera* is polyphyletic. *A. auriculata* is sister to *J. pallida* and *N. gracilis* is sister to *R. nudiflora,* and the two groups make a clade. The included species of *Merxmuellera* are resolved as paraphyletic and are inserted near the base of the tree along with *P. macrantha*

TrnL-F

Analysis of the trnL-F sequence data retrieved 491 most parsimonious trees, whose consensus is well supported at most nodes as shown in Appendix Fig. 2. *Tribolium* is paraphyletic with respect to *K. purpurea* + *K. tenella,* its relationship to the Australasian species being unresolved. *Karroochloa* is polyphyletic, since *K. schismoides* is resolved as sister to *Schismus* (bootstrap=97%). *M. guillamodae* and *P. macrantha* show an early divergence. The analysis also retrieved two strongly supported (bootstrap>86%) monophyletic sections (Uniolae and Acutiflorae) of Tribolium except that *T. pusillum* was placed within section Acutiflorae with a bootstrap percentage of 88.

TOPOLOGICAL CONFLICT AMONG THE MOLECULAR DATA SETS

When considering only the nodes that have BS > 50%, two nodes on the trnL-F tree are found to conflict with three nodes on the ITS tree, together representing two conflict areas are evident. 1. While trnL-F places *T. pusillum* as sister to *T. obliterum* + *T. acutiflorum* (bootstrap=88%), ITS resolved *T. acutiflorum* + *T. obliterum* + *T. obtusifolium* as monophyletic (bootstrap=87%) and sister to *K. purpurea* + *K. tenella* (bootstrap=80). Under ITS *T. pusillum* is resolved as sister to T. ciliare + *T. echinatum* though with poor support (bootstrap < 50%). ITS identifies *M. guillamodae* as sister to *P. macrantha* (bootstrap = 53%) whereas trnL-F identifies *M. guillamodae* as sister to all other species except *P. curvifolia* (bootstrap = 100%). Results of the Templeton test (Table 4.) show moderately significant length increases when one molecular data partition topology was constrained by the other, ITS by trn (p=0.0035) and trn by ITS (p=0.0076). Most of the increase was attributed to strongly supported nodes in trn that were constrained to weakly supported nodes in ITS and when two strongly supported nodes were contradicted. Constraining the trnL-F data to the ITS topology in relation to the node that includes *M. guillamodae* + *P. macrantha* (53% bootstrap support) result in the greatest increase in tree length (p=0.0348). For the constrained ITS tree, the most significant increase in tree length was observed when the ITS data was constrained to the node that included *T. pusillum* + *T. obliterum* + *T. acutiflorum* (bootstrap=88%).

COMBINED MOLECULAR ANALYSES

The analysis of the combined molecular data yielded a highly resolved, strongly supported tree with most of the nodes being retrieved in the bootstrap analysis.The analysis retrieved a weakly supported (bootstrap = 51%) paraphyletic *Tribolium* with respect to *K. purpurea* + *K. tenella* and the Australasian species however there were no unequivocal morphological characters that supported these relationships. Sister relationship between K. purpurea + K. tenella and section acutiflorae is characterised by a shared absence of scattered lemma hairs, a wide palea apex and lack of palea indumentum between the keels. Sections Acutiflorae and Uniolae are strongly supported monophyletic clades (bootstrap = 98% and 100% respectively). Synapomorphies for section Acutiflorae are the presence of stolons and leaf

indumentum while section Uniolae is distinctive due to the possession of a nondistichous arrangement of spikelet, shorter glumes relative to spikelet, long-acuminate glume apex, broadly clavate lemma hairs, and presence of bundle-sheath extensions. Section Triboliun is paraphyletic, however three unequivocal morphological characters differentiate this group.The synapomorphies include possession of 2-3 flowers per spikelet, long-acuminate glume apex and the presence of glume bristles. The data support the basal position of section uniolae as sister to the core *Tribolium* clade. *Karroochloa* is polyphyletic with *K. schismoides* being sister to the monophyletic *Schismus* (bootstrap=100%). Results of character optimisation showed that the inclusion of *K. schismoides* within *Schismus* species is supported by the possession of long acuminate glume apex and the presence of glume prickles. *Merxmuellera* is paraphyletic with respect to *Ps. macrantha*, and the early divergence of these basal taxa is strongly supported by the data (bootstrap 85%). *M. guillamodae* is sister to all the species (bootstrap = 89%) except *P. curvifolia*. The Australasian group form a monophyletic clade with bootstrap = 61%, however this group lack distinctive morphological characters.

MORPHOLOGICAL PHYLOGENY

Analysis of the expanded morphological data set retrieved 140 most-parsimonious trees whose consensus tree is shown in Appendix. Fig. 4. The morphological topology strongly supports the monophyly of *Tribolium* with bootstrap (bootstrap) = 93%. *Schismus* is resolved as monophyletic with a bootstrap of 54% and is resolved as sister to *Tribolium* though with weak bootstrap percentage of 57. *Karroochloa* is not resolved as either monophyletic or paraphyletic. *Merxmuellera* species and *P. macrantha* occupy the poorly resolved position outside the tree. The included Australasian species, *J. pallida*, is resolved as sister to *Karroochloa, Tribolium* and *Schismus* grouping. The analysis retrieved sections Uniolae and Acutiflorae as monophyletic, though with low support.

COMPARISON OF MOLECULAR AND MORPHOLOGICAL TOPOLOGIES

While molecular tree identifies *K. purpurea* + *K. tenella* and Australasian clade as embedded in paraphyletic *Tribolium*, morphology identifies *Tribolium* as monophyletic. While molecular data retrieved *Karroochloa* as polyphyletic morphological data does not contradict the monophyly of this genus. Morphological data does not support inclusion of *K. tenella* and *K. purpurea* or *J. pallida* within *Tribolium*.Templeton tests were performed by constraining the molecular data to overall morphological topology and to individual contradicting nodes. Significant changes in tree length resulted from constraining the molecular data to the node that comprised of eight species of *Tribolium* that make up sections Uniolae and Tribolium.

Table 4. Templeton WSR results testing for significance of conflict between the molecular data partition and molecular and morphological topologies. Constraining nodes identified by their bootstrap percentages on the respective trees.

Data set	Tree length	No. of character Changes	Z value	p value (*=significant)
ITS				
unconstrained	554	-	-	-
-constrained by				
trn 88%	525	20	-2.2937	0.0218*
trn 91%	520	20	-1.3416	0.1797
trn 99%	516	45	-0.4130	0.6796
trn				
unconstrained	245	-	-	-
-constrained by				
ITS87%	247	2	-1.414	0.1573
ITS80%	249	8	-1.414	0.1573
ITS53%	252	10	-2.1106	0.0348*
Molecular				
Unconstrained	812	-	-	-
-constrained by				
morphology89%	757	22	-0.4264	0.6698
morphology62%	801	53	-5.5815	0.0001*
morphology59	764	13	-2.4962	0.0126*
morphology93%	766	22	-2.2000	0.0278*
morphology76%	755	16	-0.0000	1.0000

DISCUSSION

The high level of resolution of the molecular tree indicates a high level of support for relationships among genera within *Rytidosperma* clade. This data set support the inclusion of Australasian species and *K. purpurea* + *K. tenella* within the *Tribolium* clade.

Molecular partitions and their combined analyses suggest that *Tribolium* is paraphyletic with respect to *K. purpurea* + *K. tenella* and Australasian species with nuclear data retrieving strong bootstrap support (94%) for this clade. Similar results have been retrieved by the nuclear data (Barker et al. 2003). The results conflict with morphology (Linder and Verboom 1996, Linder and Davidse 1997) and $rbpC_2$ (Barker et al, 2003) which support monophyly of the genus with bootstrap = 92% (morphology). The support for $rbpC_2$ result is weak as analysis was based on thin representation of the genus.

Analysis of ITS data set retrieved the three sections within *Tribolium* that have been defined by Visser and Spies (1994) and Linder and Davidse (1997) although *R. pumilum* was included at the base of section *Tribolium*. Highly supported (bootstrap percentages > 80) monophyletic sections *uniolae* and *acutiflorae* were retrieved by ITS data analysis, while a paraphyletic section *Tribolium* was retrieved.
Analysis of trnL-F data showed a high bootstrap support for the three sections within *Tribolium*. These results were in agreement with Visser and Spies (1994) cytogenetic studies that had shown section Tribolium to be paraphyletic, while sections Uniolae and Acutiflorae were monophyletic. However, the results conflict Linder and Davidse (1997) that morphological results that supported monophyly of all the sections within *Tribolium*, albeit low support. Analysis of the combined molecular data retrieved unresolved section Tribolium.

Combined molecular analysis comfirms the monophyly of *Schismus* (BS=100%), in agreement with morphology and anatomy results of Linder and Verboom (1996). *Karroochloa* is retrieved as polyphyletic and *K. schismoides* is sister to the *Schismus* clade (BS=100%). The results of Linder and Davidse (1997) morphological study

yielded a strong relationship between *K. schismoides* + *K. curva* (bootstrap = 86%) and *K. curva* might also be embedded within *Schismus*. Australasian species form a monophyletic group with moderate support (BS=69%). This might be an indication of long distance dispersal (Linder and Barker, 2000) that separated the Australasian group from the rest. The basal *Merxmuellera* group is paraphyletic.

Nuclear and plastid data disagree in their placement of *T. pusillum*. TrnL-F analysis placed *T. pusillum* within section *acutiflorae* while ITS data placed it within section *Tribolium*. The combined analysis placed these taxa outside either of the group and at the base of the core *Tribolium* clade. One possible explanation for such a pattern is that *T. pusillum* is of hybrid origin. Presence of hybrid taxa in phylogenetic studies affects the resulting phylogenetic hypothesis. McDade's (1990) investigations on the effects of hybrids on phylogenies have shown that inclusion of hybrids in phylogenetic analysis have resulted in their placement as basal clade to the lineage that include their most derived characters. This seems to be the case with *T. pusillum* in the combined molecular analysis as it comes basal to the core *Tribolium* group. This is because homoplasy results in uncertain placement of the taxa. However one expects the trnL-F, because it is non-recombining to link hybrids to their maternal parent. Linder and Davidse (1997) had referred to the possibility of hybridisation in the group retrieved by cytology studies and this involved *T. brachstachyum* with the parents possibly being *T. uniolae* and *T. hispidum*. However, cytogenetic evidence does not point to the likelihood of *T. pusillum* being a hybrid

Molecular and morphological topologies showed substantial conflict and strong support for the contradicting nodes. Morphological analysis was based on a smaller number of phylogeneticaly informative characters which yielded a tree with low resolution and poor support, and low CI value indicative of high levels of homoplasy. In contrast molecular data yielded a larger number of informative characters thus the molecular estimate represents a stronger phylogeny. Combination of the molecular and morphological data in a single analysis resulted in a topology that was at odds with both the molecular and morphology based trees. Rather than use morphological data for constructing the tree, I have used a molecular tree as a framework for interpreting morphological evolution as supported by Hedges and Maxson (1996).

Possession of long acuminate glume apex and glume prickle relates *K. schismoides* and *Schismus* species. Section Uniolae is distinctive by a few characters: nondistichous arrangement of spikelet with glumes shorter than spikelet, long-acuminate glume apex, broadly clavate lemma hairs, and presence of bundle-sheath extensions. Section Tribolium is distinguished by possession of 2-3 flowers per spikelet, long-acuminate glume apex and glume bristles. Section Acutiflorae is distinctive by two characters: presence of stolons and leaf indumentum, and this is sister to *K. purpurea* + *K. tenella* and the group is characterised by three synapomorphies: characterized by the absence of scattered lemma hairs, wide palea apex and the absence of palea indumentum between the keels.

TAXONOMIC IMPLICATIONS

Based on the current day view that formally recognised groups of species should be monophyletic (Backland and Bremer, 1998) and higher taxa should be delimited to be monophyletic. In addition, optimisation of morphological characters onto the morphological phylogeny revealed synapomorphies that characterised the groups that have emerged in the molecular analysis. Backland and Bremer (1998) have suggested that monophyletic groups should be characterised by distinctive morphological characters so that they can be identifiable. Paraphyletic groups that are supported by synapomorphic characters that have been used in taxonoming delimition in other groups should be formally described on the basis of such characters (Backland and Bremer (1998). The results of the molecular analyses lead me to make the following key suggestions regarding the taxonomy of the group: The early divergence of section *uniolae* is supported by the nuclear and plastid data sets. Adamson and Spraque (1941) had placed *T. uniolae* and *T. brachystachyum* in the genus *Plagiochloa.* Present study favours the old classification and indicates segregation of section Uniolae from *Tribolium*. The other possibility may be splitting the three sections in *Tribolium* and elevating each to generic level and assigning a new name for each section.

K. schismoides be included in *Schismus*. The name of this species suggests that even early taxonomists were aware of the stricking similarity between this species and the species of *Schismus* hence its name. Analysis that includes *K. curva* may yield more inside into the relationships in the group.

CONCLUSIONS AND FUTURE DIRECTIONS

Molecular data suggests a paraphyletic *Tribolium* and monophyletic sections within it. This may support recognition of the monophyletic sections at the generic level, as it has been suggested that paraphyletic groups should not be formally recognised in phylogenetic studies. Molecular data yields large data sets and potentially more phylogenetically informative characters. These result in trees of higher resolution and low levels of homoplasy. Combining molecular and morphological data sets resulted in a topology that supported neither of the partitions, hence this analysis was excluded. Topologies obtained from molecular partitions were relatively similar, with the difference only being in their treatment of *T. pusillum* which may be of hybrid origin.

Complete sampling of the species of *Schismus* and *Karroochloa* would clarify the relationships and probably yield robust phylogenies. Sequencing another gene region psbA-trnH might shed more light into the history of the group.

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APPENDIX.

A_auriculata	?? ??
N_gracilis	??
R_nudiflora	??
J_pallida	1000001420011-0000111000000-111111-3000
K_purpurea	1010001220011-0000100011000-01001112010
K_tenella	0010001220011-0000100011000-01000011010
K_schismoides	0010001220110-0000111010000-11111??1010
S_barbatus	0000001122110-0101101000101-00110110010
S_barbatus	0000001122110-0101101000101-00110110010
S_scaberrimus	1000001222110-0101100100001-00100111010
S_inermis	1000001222010-0101111000001-1011111?010
T_brachystachyum	10101001222010101000100010300011111 2101
T_brachystachyum	10101001222010101000100010300011111 2101
T_ciliare	00101010010010101000010001300011000 0100
T_echinatum	00101011002000101000010000310010101 1100
T_hispidum	10101011001000101000010000300011101 1100
T_pusillum	00101112002010101000110011320010100 1100
T_obliterum	11001011220110101000010000310000?110100
T_obtusifolium	1100101121??0101000010000310000011 1100
T_uniolae	10001003221110101000110010300010111 3101
T_utriculosum	00101011002001101000110010300011111 1100
T_acutiflorum	11011012220100101000010010310000011 0100
M_guillarmodae	101000041001?-00001111000?2-1A100?????0
M_disticha	101010140001?-0000120100?0-1110A?13??0
M_dura	101000041001?-0000120000?0-11110??2??0
M_stricta	1000000B1001?-0000120100?0-111A0??3010
R_pumilum	???
P_macrantha_711	100000040001?-0000121000?0-11100?03??0
P_curvifolia_594	1000001400011-0000121000000-01100102000

A={01}, B={34};

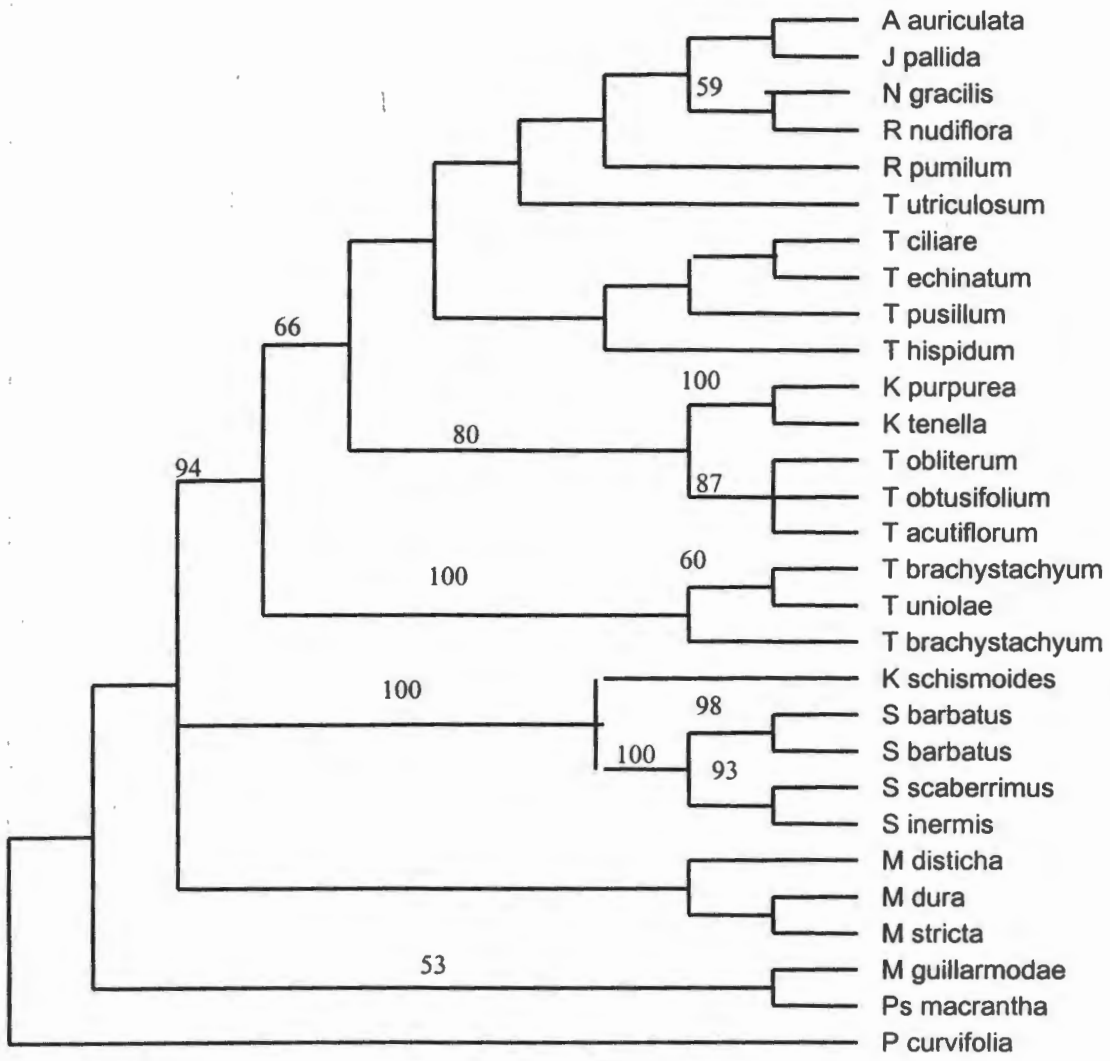

Fig. 1. Strict consensus tree of the six most parsimonious trees obtained by analysis of ITS

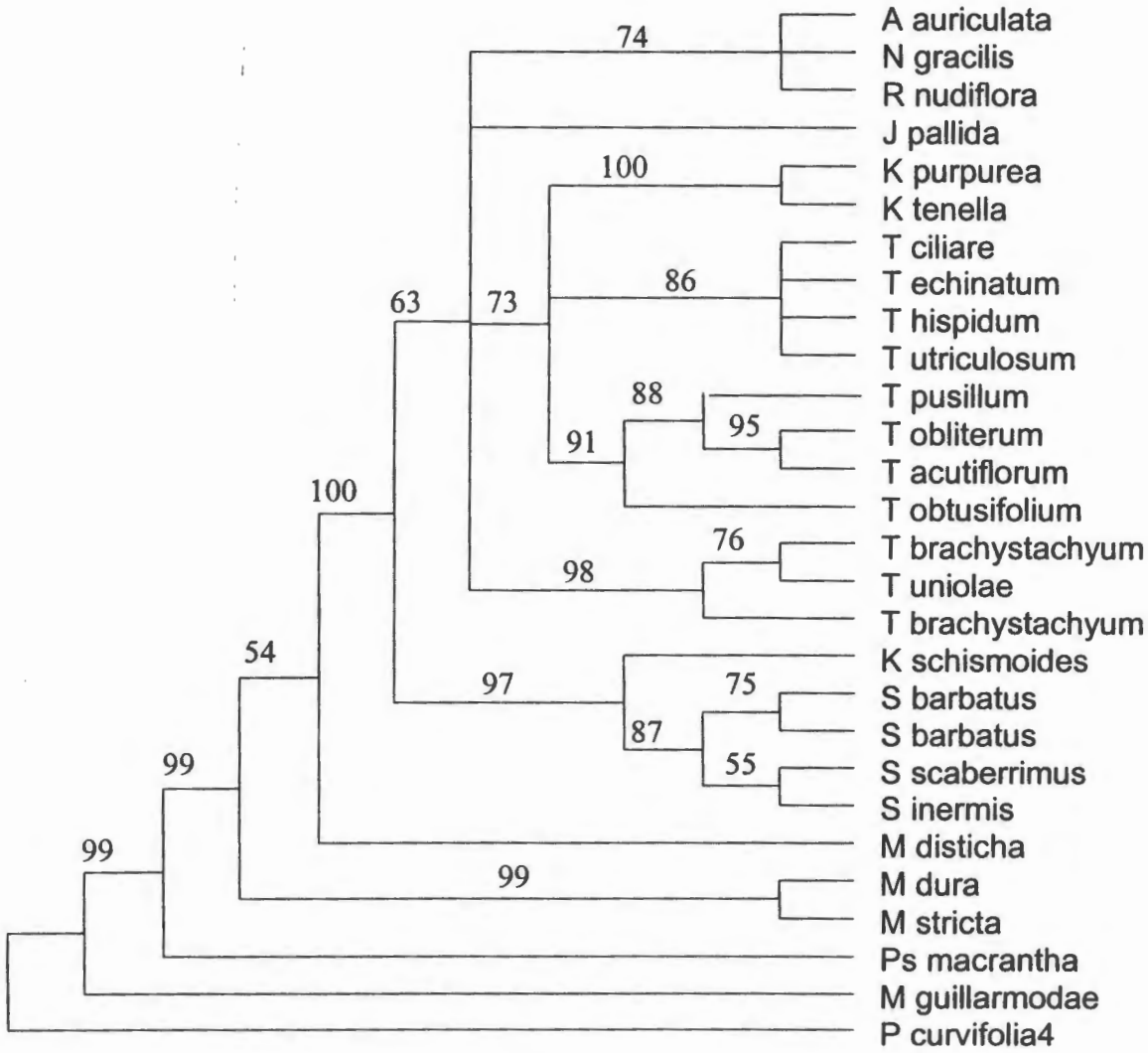

Fig. 2. Strict consensus of the 491 most parsimonious trees obtained by analysis of the TrnL-F

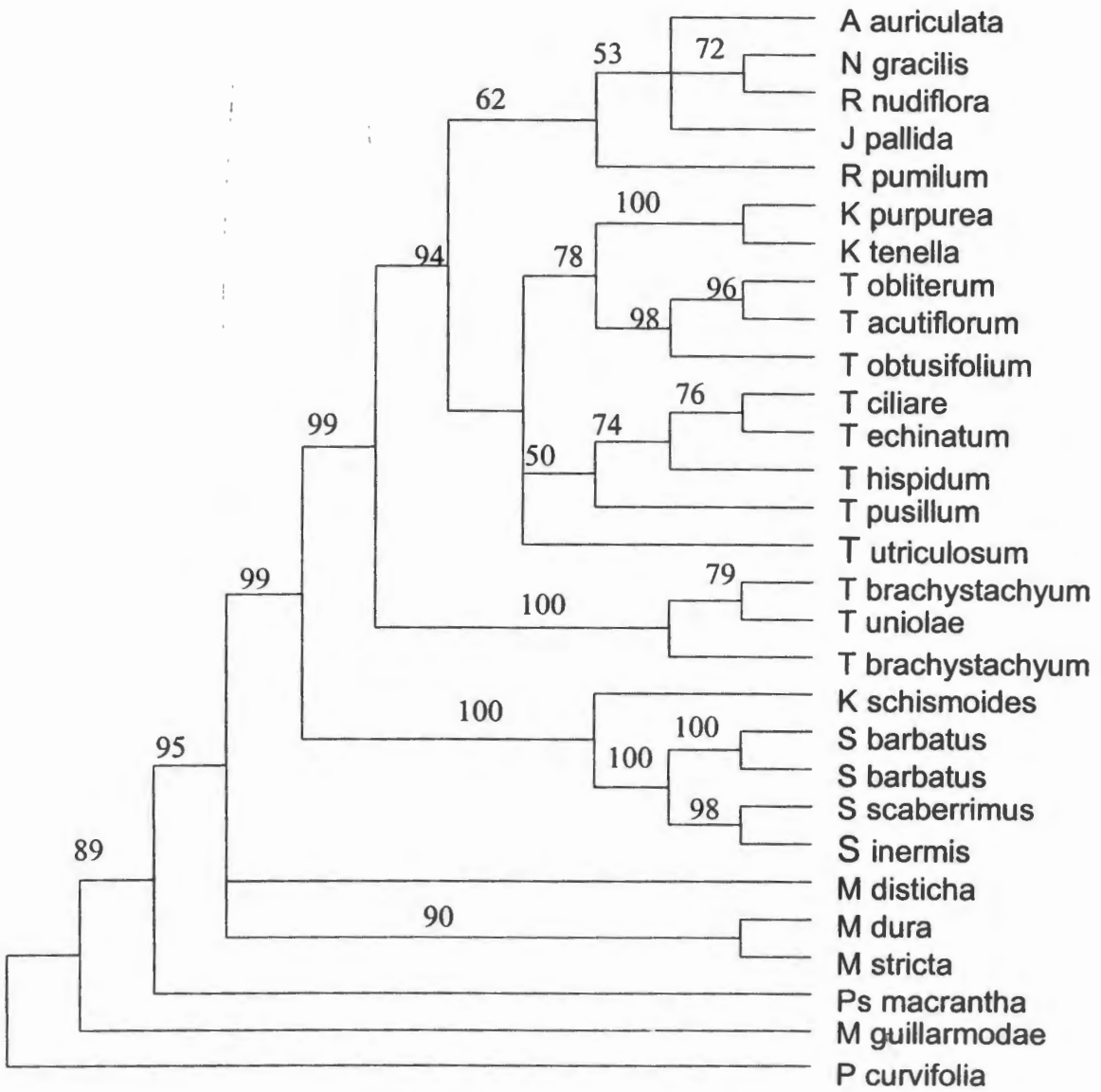

Fig. 3 Strict consensus of the 12 most-parsimonious trees obtained by analysis of the combined molecular data

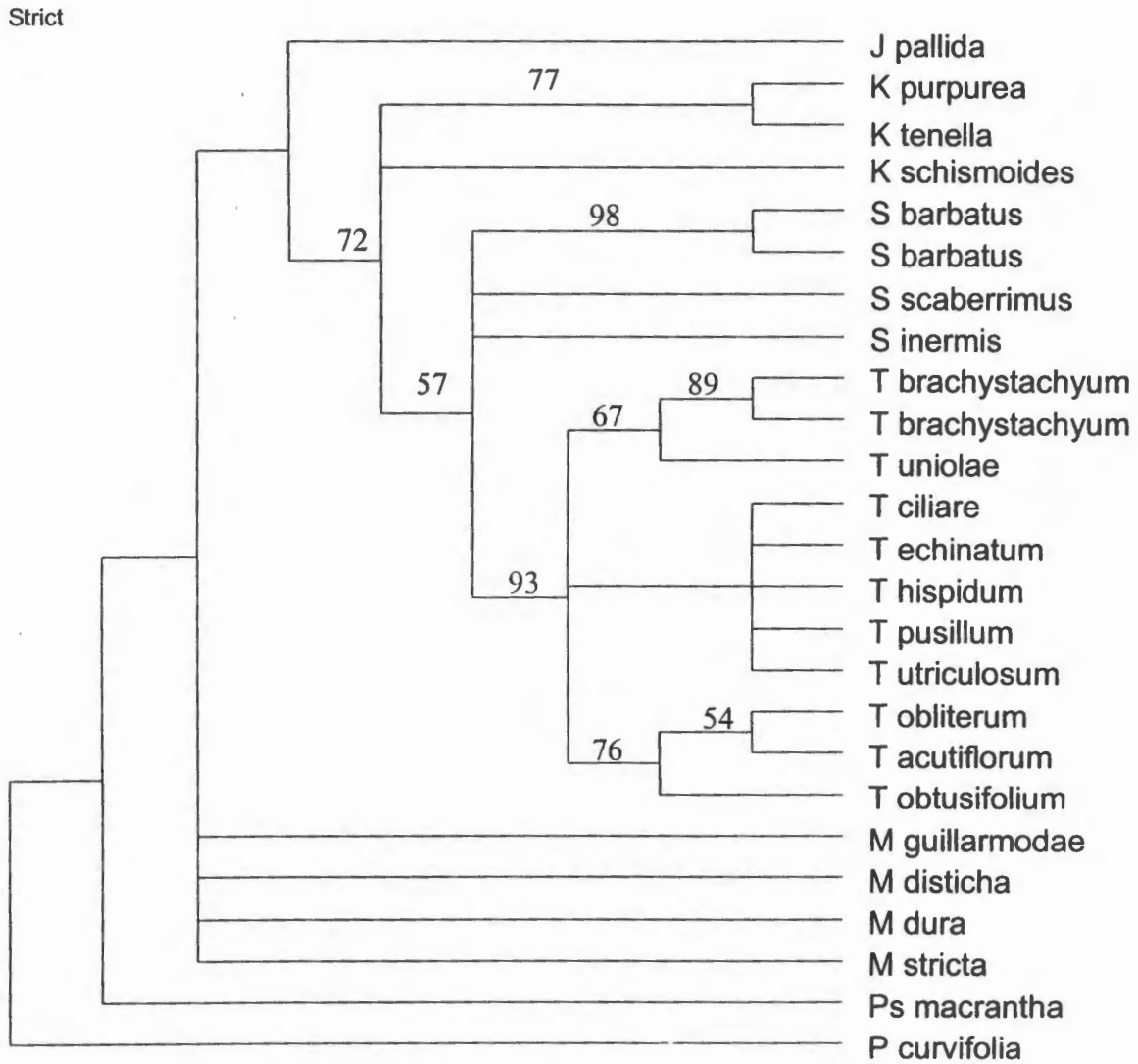

Fig. 4. Strict consensus of the 140 trees obtained by analysis of the morphological data